FIRST ANNUAL REPORT (YEAR 1)

for period September 1, 1994 to August 31, 1994

Report Date: October 31, 1994

ONR Grant No. N00014-93-J-1034
(ECU Grant #5-01071)

PRECLINICAL INVESTIGATION OF LYOPHILIZED PLATELET PREPARATIONS

Principal Investigator: Arthur P. Bode, Ph.D.
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Attachments:

1. Report from subcontract principal investigator, Marjorie S. Read, Ph.D., The University of North Carolina at Chapel Hill.

2. Abstract to be presented at annual meeting of American Association of Blood Banks, Nov. 12-17, 1994, San Diego, CA.

3. Abstract to be presented at annual meeting of American Society of Hematology, Dec. 2-6, 1994, Nashville, TN.

Administrative Activity:

Because of the rush in processing and activation of this proposal, the animal studies and proper billing for personnel were somewhat delayed in getting started. However, all specific aims were initiated in the first year, and progress was made on several points. Specific Aim #5 (gel studies of internal signs of platelet activation, to be done by a collaborating lab at ECU) has been modified due to the inability of the collaborating lab to finish the work. The technician involved had a serious medical emergency necessitating work stoppage. The goal of showing activation of lyophilized platelets through gel studies will be completed in part by the subcontractors at UNC, but we will seek permission to use the Year 2 resources budgeted initially for this aim to develop an adjunct to the animal studies; we will study the hemostatic efficacy of human lyophilized platelets in our established model of dogs with a platelet dysfunction induced by prolonged extracorporeal circulation of blood. Approval of the ONR Scientific Liaison Officer for this change in workplan will be officially petitioned as soon as the modification in clinical protocol is approved by the ECU Animal Subject Review Board.

A preliminary summary of data from this project was presented on May 18 at the ONR complex in Alexandria, VA, to a meeting of ATD project directors and participants. Also present were representatives of Armour Pharmaceutical Corp. to report on their plan to pursue commercial production of lyophilized platelets by our technique(s) for human trials as soon as possible. A progress report was given also by this PI at the annual meeting of the British Blood Transfusion Society at Southampton, England, Sept. 13, 1994. Data from the first year of studies will appear in an oral presentation to be given by the PI at the annual meeting of the American Association of Blood Banks, Nov. 12-17, 1994, at San Diego, CA, and also in a poster at the annual meeting of the American Society of Hematology last year, Dec. 1993, at St. Louis, MO.

The Clot Signature Analyzer purchased in Year 1 was late in delivery, but has been pressed into hard service in the latter half of Year 1. We have experienced sporadic downtimes, but usually with quick repair and good support from Xylum Corporation. The CSA is now used in the studies of hemostasis during infusions of reconstituted canine platelets in dogs on heart-lung bypass (Specific Aim #4), as well as to characterize canine and human platelet preparations prior to further experimentation. This is a prototype unit that will be swapped out for the production model as soon as the new one is released for use by the FDA. The purchase is being handled as a one-year lease with monthly payments totalling the F.O.B. price; the exchange for the new model will be without extra cost.

Scientific Progress:

As detailed in the interim reports, the main emphasis in workplan at ECU for Year 1 was in the production and testing of canine lyophilized platelets. After high yield preparations were obtained routinely, we initiated animal studies under Specific Aim #4 in the dog heart-lung bypass model. Thirteen in vivo studies have been performed in Year 1, using three sets of clinical conditions to discern the effect of

infused lyophilized platelets on the compromised hemostatic system of dogs on prolonged cardiopulmonary bypass. In the first 4 studies, the dog was placed on the extracorporeal circulation pump for 2 hrs and then infused with 2-3 x 10^9 reconstituted lyophilized heterologous canine platelets. Hemostasis was evaluated with bleeding time measurements performed by needle puncture of the ear (microvascular injury) or of the jugular vein (circulatory thrombosis). The infused platelets clearly shortened the bleeding time (from > 12 minutes down to 2-5 minutes) over controls of saline or plasma infusion. In the next 7 studies, an antifibrinolytic drug, AMICAR (1g IV),was given after 1 hour on the pump, prior to infusion of reconstituted platelets. Again, the dogs in which platelets were infused showed much lower bleeding times than the controls. The CSA was available for some of these studies and showed a very significant shortening of the in vitro bleeding time and the collagen-induced thrombus formation time as a result of platelet infusion. The final set of studies is still underway with the added feature of a plasma infusion after the AMICAR prior to giving the platelets to show the added benefit of the platelets over AMICAR and plasma alone. In the first two dogs in this group, the data suggest that the combination of AMICAR, plasma, and platelets can bring the bleeding times down all the way to presurgical baseline values. Controls are now being conducted. Repeating tests after the dogs are taken off bypass and weaned from the pump has shown persistence of the hemostatic effect of infused platelets for three hours or more. We regard these findings as strongly supporting the notion that lyophilized platelets may be an effective form of transfusion medicine in hemorrhagic conditions caused by platelet dysfunction.

Because of the problems encountered in conducting the SDS-PAGE analysis of markers of activation in lyophilized platelets, we turned to a flow cytometric analysis in another system. In the project supported by grant N00014-92-J-1244, we found that lyophilized platelets adhered to denuded blood vessel strips in the Baumgartner perfusion chamber. Under N00014-93-J-1034, the nonadherent platelets were examined for evidence of activation in terms of expression of neoantigens CD62 and GP53 (CD63) and released Thromboxane (B_2). We found that on average the PARA21 lyophilized platelets showed a twofold increase in CD62 and GP53 expression and a fivefold increase in TxB_2 after the Baumgartner perfusion exposure. We will continue to characterize this response to document evidence of the ability of lyophilized platelets to activate in response to physiologic stimulation. A manuscript on this data has been prepared for submission to Blood.

In the second year we will start the second transfusion model at ECU (ruptured pig aorta repair) and expand the canine bypass model to include infusions of human platelet preparations. Work has already begun on processing donor pig platelets for lyophilization to create a stockpile for later use in the aorta repair model. For the experimentation with human platelets in the canine transfusion model, we will first have to modify the animal subject protocol and receive IRB and Navy approval. The initial experiments will involve non-dried platelets to establish parameters such as recovery and survival of xenographic cells in the dog. Dr. Read and colleagues will continue their studies on immunogenicity of the lyophilized platelets in multiple infusions and on bacteriostatic properties of the processing. We hope that the progress thus far is judged to be satisfactory.

Annual Report
University of North Carolina at Chapel Hill

Contract: UNC/ECU
Grant No. N00014-93-1034
The Office of Naval Research
Department of the Navy

Performance Site: University of North Carolina at Chapel Hill
Principal Investigator: Marjorie S. Read, Ph.D

Grant No. N00014-93-1034
Annual report

The biochemical and thrombogenic effects of rehydrated platelets and the characterization of the surface antigens of rehydrated platelets (Specific aim #1)

We are continuing our studies of rehydrated human and canine platelets using SDS-PAGE and immunoblotting of human and canine platelet lysates and releasates. Lysates and releasates of rehydrated platelets were prepared as previously described (see progress report for October 1993-December 1993). Commercially available antibodies were used to probe Western blots of lysates and releasates for von Willebrand factor (vWF) (DAKO), actin (Sigma Immunochemicals), thrombospondin (AMAC, Inc.), GPIb (DAKO and AMAC, Inc.), fibrinogen (Calbiochem Corp.), and fibronectin (Calbiochem Corp.).

The vWF antibody reacted with a high molecular weight protein band present in western blots of both fresh and rehydrated human and canine platelets. The actin antibody reacted with a low molecular weight band present in western blots of both fresh and rehydrated platelet lysates and releasates (both human and canine). The thrombospondin antibody did not react well with any protein in the rehydrated platelets, and only minimally reacted with a protein in the fresh platelet preparation. The DAKO antibody to GPIb reacted minimally with a high molecular weight protein on Western blots of fresh platelets without any visible reaction with Western blots of rehydrated platelet preparations. The AMAC antibody reacted strongly with a high molecular weight protein in the fresh platelet lysate, and to a lesser degree with a protein of identical molecular weight in the rehydrated platelet lysate. The antibody to fibrinogen reacted strongly with several protein bands in Western blots of fresh platelet lysates, with less reactivity in Western blots of rehydrated platelets. When the antibody was preabsorbed with excess purified fibrinogen, the bands disappeared, suggesting that they were specific for fibrinogen, and represented fragments of the fibrinogen molecule. The antibody to fibronectin reacted with a high molecular weight protein in western blots of fresh platelets, but did not react with any proteins in Western blots of rehydrated platelets. These experiments are being repeated with the Amersham ECL chemiluminescence system to enhance the sensitivity of the assays. We suspect that a more sensitive detection system will reveal reactivity of some of these antibodies with rehydrated platelet preparations on Western blot.

The characterization of human and canine rehydrated platelets using light microscopy is continuing in our laboratory. For these studies, rehydrated platelets and fresh platelets are allowed to spread on a glass slide for 5 minutes-4 hours. The spread platelets are then fixed onto the slide and treated with detergent to permeabilize the cell membrane. For the visualization of filamentous actin within the platelets, FITC- or TRITC-labeled phalloidin is incubated with the platelets. For the visualization of other platelet proteins, antibodies specific for these proteins are incubated with the platelets. A secondary antibody labeled with fluorescein or rhodamine is used to visualize the localization of the antibodies in the platelets. We have used TRITC-phalloidin (Molecular Probes) and FITC-phalloidin (Sigma Immunochemicals) for labeling filamentous actin. Antibodies to GPIb (DAKO and AMAC, Inc.), GPIIbIIIa (AMAC, Inc.), myosin (Sigma Immunochemicals), tropomyosin (Sigma Immunochemicals), and fibrinogen (Organon Technika) are also being used. Actin arrangement in rehydrated platelets appears similar to that seen in fresh platelets. In fresh and rehydrated platelets fixed onto the slide after 5 minutes, actin staining appears at the center of the discoidal platelets. As expected, rehydrated platelets appear to spread at a slower rate than fresh platelets. At 2 hours, fresh platelets form a 'mesh' over the surface of the slide. The majority of rehydrated platelets at 2 hours remain as single platelets. Although the rehydrated platelets are not completely spread, the platelets appear 'flattened' with psuedopods present. Actin staining was seen in the psuedopods of both fresh and rehydrated platelets. With complete spreading, actin appears throughout the platelets in both fresh and rehydrated samples. Antibodies to GPIb and GPIIbIIIa reacted with a protein on the surface of platelets from fresh and rehydrated platelet preparations. The staining pattern for these proteins appears to be similar in fresh and rehydrated platelets throughout the spreading process. Antibodies to myosin and tropomyosin are also being used in these studies. Myosin antibodies reacted with a protein in the cytoplasm of rehydrated and fresh platelets. The staining appears in the central region of the platelets in both fresh and rehydrated samples at both 5 minutes and 2 hours. Initial data using antibodies to tropomyosin show reactivity with a protein that forms a 'loop' in the platelet cytoplasm of rehydrated and fresh samples. Using an antibody to fibrinogen, we find staining on the surface of rehydrated and fresh platelets in a punctate pattern which becomes more diffuse with platelet spreading. The patterns appear similar in both fresh and rehydrated platelets. We are continuing our studies using these antibodies. Although these experiments are

currently being used to characterize the rehydrated platelets, they might lead to the future development of a quality control test for rehydrated platelet preparations.

The effects of multiple infusions of rehydrated platelets in the canine animal model (Specific aim #2)

We have used an ELISA to determine if any animals that have received rehydrated platelets has developed an antibody to the rehydrated platelets. We have infused 4 dogs with rehydrated canine platelets multiple times. Each dog was infused with an infusate composed of a pool of platelets from appoximately 3 donor dogs. We have tested the serum against both fresh and rehydrated platelets preparations from each dog. In previous experiments, one multiply infused dog appeared to have an antibody against one component of the rehydrated platelet infusate. However, re-evaluation of that data revealed a high background signal. Further testing using whole and lysed fresh and rehydrated platelets from the donor dogs does not show evidence of antibody production in the infused dog. Our current data suggests that multiple transfusions of rehydrated platelets has not led to a significant increase in the immune response of transfused animals.

The effects of the stabilization and lyophilization method on bacterial contamination in the preparation of platelets. (Specific aim #3)

We have started investigating the effects of our stabilization and lyophilization process on bacterial contamination in our platelet preparations. To date, we have cultured platelet samples from two preparations for bacterial contamination. Neither preparation had bacterial growth after 6 days. We are continuing these studies to insure sterility in preparations. The next studies will involve innoculation of the platelet preparations with bacterial contaminants to investigate the bactericidal properties of paraformaldehyde and lyophilization.

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HEMOSTATIC PROPERTIES OF HUMAN LYOPHILIZED PLATELETS IN A THROMBOCYTOPENIC RABBIT MODEL AND A SIMULATED BLEEDING TIME DEVICE.

A. P. Bode, M. Blajchman, L. Bardossy*, and M.S. Read, Departments of Pathology, East Carolina University, Greenville, NC, and McMaster University, Hamilton, Ontario, and University of North Carolina at Chapel Hill, NC.

We have shown previously that our preparations of lyophilized human platelets (Lyo-Plt) are structurally intact upon reconstitution, and that they adhere to thrombogenic surfaces in vitro and in vivo (Blood 82:159a, 1993). Now we have tested the ability of these preparations to correct the ear bleeding time (BT) in rabbits made thrombocytopenic and immunosuppressed as detailed elsewhere (Blood 82:3489, 1993). Also, we have measured the in vitro bleeding time (IVBT) and collagen-induced thrombus formation (CITF) of Lyo-Plt in a prototype in vitro bleeding time device called the Clot Signature Analyzer (CSA, Xylum Corp, NY). In the rabbits, the endogenous platelet count was $\leq 10 \times 10^6$/mL and the BT was ≥ 900 seconds before infusion of 40-50 x 10^9 platelets. A platelet count and duplicate BTs were then performed one hour after infusion. For the CSA test, platelets were resuspended in fresh citrated plasma at 300-500 x 10^6/mL and combined with an equal volume of washed RBC to remake whole blood. $CaCl_2$ was added to 5 mM in the blood just prior to initiating each run. Mean results for Lyo-Plt versus human platelet-rich plasma (fresh) or 5-8 day old expired blood bank platelet concentrates (Exp PC) are tabulated below:

	Rabbit Model				Clot Signature Analyzer		
	n	% Recovery	Count	Ear BT	n	IVBT	CITF
Lyo-Plt	(12)	58%	108	234 sec.	(4)	118 sec.	73%
Fresh	(6)	79%	153	177 sec.	(4)	134 sec.	88%
Exp PC	(0)	ND	ND	ND	(3)	281 sec.	39%

The Lyo-Plt had reduced 1 hour recovery in the rabbit model relative to fresh platelets, but the mean BT result was similar to the value seen in non-infused rabbit controls with equivalent endogenous platelet counts. In the CSA, the Lyo-Plt gave results similar to that of fresh platelets and better than that of stored platelets in the IVBT and the CITF (t-test, $p < 0.05$). These findings support the notion that Lyo-Plts are hemostatically active.

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AABB Nov 12-17, 1994

HEMOSTATIC PROPERTIES OF LYOPHILIZED PLATELETS IN TESTS OF BLEEDING TIMES.

Arthur P. Bode, Marjorie S. Read, Robert M. Lust. Depts. of Pathology and Surgery, East Carolina Univ., Greenville, NC, and Dept. Pathology, Univ. of N.C. at Chapel Hill.

Background: We have previously shown that lyophilized platelets (L-Plt) retain properties of adhesion and activatability in the Baumgartner perfusion chamber (Trans 33:72S, 1993). Now we have analyzed L-Plt in two systems directly testing hemostatic function. **Study Design:** One is a prototype device simulating the Ivy bleeding time in vitro (IVBT) and collagen-induced thrombus formation (CITF) in recalcified whole blood (Xylum Clot Signature Analyzer: CSA); the other is an in vivo bleeding time in dogs on full clinical heart-lung bypass before and after infusion of L-Plt. **Results:** On the CSA, L-Plt gave an average (n=4) IVBT of 1 min 58 sec and a CITF of 73% versus 2 min 14 sec and 88% respectively for fresh platelets. Expired platelet concentrates gave indeterminate results because aggregates clogged the lines. IVBT > 6 min and CITF < 25% is typical of vWD patients. In two canine heart-lung bypass studies, the in vivo bleeding time improved from >15 min to 5-7 min after infusion of a bolus of 2-3 x10^{11} L-Plt. The corrected count increments were (#1) 88% and (#2) 47% based on estimated circulatory volume. **Conclusions:** These results demonstrate the hemostatic activity of L-Plt and their potential value in transfusion medicine.

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